Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection

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SUMMARY

Chronic viral infections often result in T cell exhaustion. To determine the molecular signature of exhaustion, we compared the gene-expression profiles of dysfunctional lymphocytic choriomeningitis virus (LCMV)-specific CD8+ T cells from chronic infection to functional LCMV-specific effector and memory CD8+ T cells generated after acute infection. These data showed that exhausted CD8+ T cells: (1) overexpressed several inhibitory receptors, including PD-1, (2) had major changes in T cell receptor and cytokine signaling pathways, (3) displayed altered expression of genes involved in chemotaxis, adhesion, and migration, (4) expressed a distinct set of transcription factors, and (5) had profound metabolic and bioenergetic deficiencies. T cell exhaustion was progressive, and gene-expression profiling indicated that T cell exhaustion and anergy were distinct processes. Thus, functional exhaustion is probably due to both active suppression and passive defects in signaling and metabolism. These results provide a framework for designing rational immunotherapies during chronic infections.

INTRODUCTION

After acute infection, naive antigen-specific CD8+ T cells become activated, proliferate, acquire effector functions, and differentiate into effector CD8+ T cells. Most effector CD8+ T cells die by apoptosis, but ~5%–10% survive and differentiate into memory CD8+ T cells. These memory CD8+ T cells downregulate some properties of effector CD8+ T cells but retain the capacity to rapidly reactivate effector functions upon antigen encounter (Wherry and Ahmed, 2004). Memory T cells also undergo rapid proliferation upon re-exposure to antigen. In addition, memory T cells can survive and persist long term in the absence of antigen and acquire the ability of “self-renewal” by undergoing homeostatic proliferation in response to IL-7 and IL-15 (Surh et al., 2006; Wherry and Ahmed, 2004). This combination of antigen-independent persistence and the ability to rapidly reactivate effector functions allows memory CD8+ T cells to confer robust long-term protective immunity.

During many chronic infections, in contrast, severe defects in CD8+ T cell responses develop, and virus-specific CD8+ T cells often fail to differentiate into memory CD8+ T cells (Shin and Wherry, 2007). CD8+ T cell exhaustion was first described with chronic LCMV infection of mice during which virus-specific CD8+ T cells persist but lack effector function (Zajac et al., 1998). During chronic LCMV infection, virus-specific CD8+ T cells initially develop the ability to perform effector functions. These functions are lost, however, in a hierarchical manner during chronic infection with some functions that are exhausted early (e.g., IL-2, cytotoxicity, and proliferation), whereas others (e.g., IFN-γ) persist longer (Wherry et al., 2003). Eventually, especially if viral load is high and CD4+ help is lacking, virus-specific CD8+ T cells fully lacking effector functions are found (Fuller and Zajac, 2003; Ou et al., 2001; Wherry et al., 2003). Similar types of dysfunction have also been described in other experimental models of viral infection, during human chronic infections and during cancer (Shin and Wherry, 2007).

CD8+ T cell exhaustion during persisting infection is likely to have an important impact on viral control, and some of the underlying mechanisms for this dysfunction are beginning to be elucidated. Recently, we have found that the inhibitory receptor PD-1 is an important pathway regulating CD8+ T cell exhaustion during chronic LCMV infection in mice (Barber et al., 2006). Blockade of the PD-1:PD-L pathway during chronic LCMV infection leads to recovery of T cell function and reduced viral load (Barber et al., 2006). These results have been extended to primates and humans in which the PD-1:PD-L1 pathway also regulates function of SIV-, HIV-, and HCV-specific T
This approach revealed key insights into the biology of pattern of transcription factors. Fifth, CD8+ T cell exhaustion, we compared the gene-expression profiles of virus-specific exhausted CD8+ T cells.

We have examined the molecular signature of CD8+ T cell exhaustion by using the well-characterized model of chronic LCMV infection. We have compared the gene-expression profiles of exhausted LCMV-specific CD8+ T cells to those of functional LCMV-specific effector and memory CD8+ T cells generated after acute infection. This approach revealed key insights into the biology of CD8+ T cell dysfunction during chronic viral infection. First, exhausted CD8+ T cells overexpressed multiple cell-surface inhibitory receptors, in addition to PD-1. Second, the fact that transcription of genes encoding molecules involved in signaling from TCR and cytokine receptors was downregulated probably compromised the efficiency of these pathways. Third, many genes for chemotaxis, migration, and adhesion were changed in exhausted CD8+ T cells. Fourth, exhausted CD8+ T cells displayed a dramatically altered pattern of differentiation compared to effector and memory CD8+ T cells including a distinct expression pattern of transcription factors. Fifth, CD8+ T cell exhaustion was associated with profound translational, metabolic, and bioenergetic deficiencies. Finally, exhausted CD8+ T cells possessed a gene-expression profile distinct from that reported for anergic T cells. It is likely that the defects in effector functions in exhausted CD8+ T cells are due to both active suppression, for example by inhibitory receptors, and passive defects in signaling and metabolism. The defects and altered pathways identified in exhausted CD8+ T cells provide an important framework from which to begin dissecting the control of CD8+ T cell differentiation when pathogens persist and suggest novel potential targets of therapeutic intervention during chronic infections.

RESULTS

Function and Phenotype of Exhausted CD8+ T Cells
To address the molecular mechanisms of CD8+ T cell exhaustion, we compared the gene-expression profiles of exhausted LCMV-specific CD8+ T cells from chronic infection to fully functional LCMV-specific effector and memory CD8+ T cells generated after acute LCMV infection. Infection with the Armstrong strain (Arm) of LCMV causes an acute infection that is cleared by day 8–10 p.i., resulting in the generation of functional effector and memory CD8+ T cells (Ahmed et al., 1984). In contrast, the clone 13 strain of LCMV causes a chronic infection with loss of effector functions (exhaustion) and ineffective viral control (Ahmed et al., 1984; Wherry et al., 2003; Zajac et al., 1998). In the present study, we have used chronic LCMV infection of CD4+ T cell-depleted mice because these conditions result in the most profound CD8+ T cell exhaustion and we wanted to gain an understanding of the molecular pathways involved in highly dysfunctional virus-specific CD8+ T cells. The main features of exhaustion, however, are preserved in CD4+ T cell-sufficient mice (Wherry et al., 2003; Zajac et al., 1998). LCMV Arm infection or clone 13 infection resulted in similar viral replication in the spleen 3 days p.i. (Figure 1). LCMV Arm is completely cleared by day 8–10 p.i., whereas replication of clone 13 continues at a high level for life in CD4+ T cell-deficient mice ([Figure 1] and Zajac et al. [1998]). In contrast to functional effector and memory CD8+ T cells generated after Arm infection, exhausted CD8+ T cells during clone 13 infection were nonfunctional and unable to produce substantial levels of IFN-γ and TNF-α upon peptide stimulation (Figures 1B–1D). It is important to point out that the studies of gene-expression profiling described below were performed on antigen-specific effector, memory, and exhausted CD8+ T cells of the same antigen specificity (e.g., the DbGP33 LCMV epitope).

Patterns of Gene Expression
To define the molecular signature of CD8+ T cell dysfunction during chronic viral infection, we profiled the gene-expression patterns of effector, memory, and exhausted CD8+ T cells by using Affymetrix U74A microarrays containing ~12,500 mouse genes. The gene-expression profiles of sorted effector, memory, and exhausted CD8+ T cells were each compared to naive CD8+ T cells so that direct comparisons between all three groups could be facilitated. An example of presort and postsort analysis of exhausted CD8+ T cells is shown in Figure 2A. A total of 490 genes was upregulated or downregulated by at least 2-fold in the exhausted CD8+ T cells (Figure 2B). The vast majority of genes (338) were differentially expressed only in the exhausted CD8+ T cells.

There was more similarity in gene expression between exhausted and effector CD8+ T cells compared to exhausted and memory CD8+ T cells as revealed by both numerical representation of genes differentially expressed (Figure 2B) and gene clustering analyses (Figures 2C and D and Figure S1 in the Supplemental Data available online). This clustering analysis, however, also reveals a number of genes uniquely upregulated (n = 123) or downregulated (n = 135) in exhausted CD8+ T cells, indicating that CD8+ T cell exhaustion reflects a unique state of gene expression compared to that of naive, effector, and memory CD8+ T cells.

A gene-expression signature could also be identified that uniquely defined naive, effector, memory, and exhausted CD8+ T cells (Figure 2E). Some expression of the genes found in the effector signature was noted in the exhausted CD8+ T cells. The panel of genes that identified exhausted CD8+ T cells, however, was not present in...
any of the other three cell types, supporting the notion that exhaustion is a unique state of CD8+ T cell differentiation. An advantage of the approach used in Figure 2E is that we isolated LCMV-specific CD8+ T cells at multiple time points after acute infection to gain a kinetic portrait of gene expression during memory CD8+ T cell differentiation similar to previous studies (Kaech et al., 2002). With this analysis, it is clear that the similarities between exhausted and effector CD8+ T cells do not persist after day 8 of acute infection, suggesting that the patterns of memory T cell differentiation and exhaustion diverge or differ substantially as functional CD8+ T cells differentiate into memory CD8+ T cells.

**Gene-Expression Signatures of CD8+ T Cell Exhaustion**

To begin to identify the molecular pathways associated with CD8+ T cell exhaustion, we identified genes that differed in expression by more than 2-fold by microarray analysis. (Table 1 and Tables S2–S5). The gene-expression profiles of exhausted CD8+ T cells were also compared directly to that of effector (Table S2) and memory (Table S3) CD8+ T cells. A subset of samples was also hybridized to the U74Bv2 and U74Cv2 Affymetrix microarrays containing an additional ~25,000 mouse genes and ESTs (Table S4). The major findings from the data shown in Table 1 and Tables S2–S5 are outlined below.

**Overexpression of Inhibitory Receptor Genes during Exhaustion**

One of the more striking results from the gene-expression profiles was the overexpression of mRNA for cell-surface molecules known or suspected to have inhibitory activity. We have recently identified PD-1 as a major cell-surface inhibitory receptor capable of regulating CD8+ T cell exhaustion (Barber et al., 2006). In the current analysis, in addition to PD-1, 13 other potentially inhibitory cell-surface pathways were identified, and most of these molecules were specifically overexpressed in exhausted CD8+ T cells (Table 1 and Tables S2, S3, and S5). These inhibitory receptors included those with well-described inhibitory function such as 2B4 (Assarsson et al., 2005), Ly49 family members (Klra7 also known as Ly49D-GE, Klra3 also known as Ly49c) (Lanier, 1998), and GP49B (Katz et al., 1996; Rojo et al., 1997). Molecules with less well-characterized but potential inhibitory capacity were
also overexpressed by exhausted CD8+ T cells including CD160 (Maeda et al., 2005), Ptger4 (Kabashima et al., 2002), and LAG-3 (Workman et al., 2002). The CD28:CTLA-4 family contains three related inhibitory receptors that can be expressed by T cells: CTLA-4, PD-1 and BTLA (Greenwald et al., 2005).
identified in the current analysis as one of the most overexpressed inhibitory receptors by exhausted CD8+ T cells (Table 1 and Tables S2 and S3) consistent with our previous studies (Barber et al., 2006). CTLA-4 mRNA was also overexpressed by exhausted CD8+ T cells, but it is worth pointing out that blockade of CTLA-4 during chronic LCMV infection had little impact on viral load or CD8+ T cell responses (Barber et al., 2006). It is interesting that the inhibitory molecule BTLA (Watanabe et al., 2003) did not appear to be upregulated by exhausted CD8+ T cells (Table S5). Thus, within the CD28:CTLA-4 family there appears to be selective expression or utilization of inhibitory receptors on CD8+ T cells during chronic viral infection.

Many of these molecules contain immunotyrosine inhibitory motifs ITIM, immunotyrosine switch motifs (ITSMs), or both, that can deliver negative signals. For others, the mechanism of signaling is less clear and, in some cases, the available data are conflicting on whether the receptor is activating or inhibitory (e.g., CD160 and 2B4). Of the inhibitory receptors identified by gene-expression profiling, all but two, KLRG1 and KLRA9, are strongly biased or uniquely expressed by exhausted CD8+ T cells.
compared to effector and memory CD8\(^+\) T cells. It will be important to determine whether these inhibitory receptors impact specific and distinct CD8\(^+\) T cell functions or to what extent the inhibitory signals from the pathways might overlap.

**Changes in Signaling Potential during Exhaustion**

Several genes encoding key signaling molecules were also differentially expressed by exhausted CD8\(^+\) T cells. Lck and NFATC2 were transcriptionally downregulated in exhausted CD8\(^+\) T cells, whereas Fyn was upregulated in both exhausted and effector CD8\(^+\) T cells (Table 1 and Tables S2 and S3). In addition, Dgk\(x\) was downregulated, whereas the related Dgk\(\gamma\) was upregulated (Table 1 and Tables S2, S3, and S5). Genes involved in calcium binding (up: s100a6, s100a4, s100a11, and s100a13) and Map kinase signaling (up: Map3k4; down: Map4k4 and Mapk8) were also modulated in exhausted CD8\(^+\) T cells. The phosphatase Shp1 was transcriptionally reduced in exhausted CD8\(^+\) T cells, and this observation might be relevant for the function of some inhibitory receptors or attenuation of TCR signaling.

A number of genes involved in cytokine signaling were also altered in exhausted CD8\(^+\) T cells. Cytokine receptor transcripts were altered including both chains of the TNFR (p55 and p75), the IL-4R\(\alpha\) (CD124), and IL-17R. When compared directly to effector or memory CD8\(^+\) T cells, IL-18R1 was also downregulated in exhausted CD8\(^+\) T cells as were signaling components of the IL-18 and IL-6 receptors pathways (IL-18Rap and IL-6st). It is also worth noting that IL-10 and IL-10R1 mRNA expression was not different among effector, memory, and exhausted CD8\(^+\) T cells (data not shown).

A major defect in CD8\(^+\) T cells during chronic viral infections is the failure to develop self-renewal and optimal responsiveness to the homeostatic cytokines IL-7 and IL-15 (Shin and Wherry, 2007). Previous studies demonstrated a defect in expression of IL-7 and IL-15 receptors during chronic viral infections, but it was unclear whether receptor expression was the only deficiency in IL-7 and IL-15 responsiveness in these virus-specific CD8\(^+\) T cells. IL-7, IL-15, and other gamma-chain cytokine signals in T cells are mediated predominantly by the kinases Jak1, Jak3, and Stat5. The gene-expression profiles of exhausted CD8\(^+\) T cells confirmed the low expression of CD127 during exhaustion (Table 1 and Table S3) but also indicated that the deficiencies in cytokine signaling are probably not confined to expression of cell-surface receptors. Although Jak3 message was increased in exhausted CD8\(^+\) T cells compared to effector or memory CD8\(^+\) T cells (Tables S2 and S3), both Jak1 and Stat5b transcription were reduced (Tables S2 and S5). Indeed, we have recently found that virus-specific CD8\(^+\) T cells generated during chronic infection are dependent on viral antigen rather than IL-7 and IL-15 for persistence (Shin et al., 2007). The observations from the gene-expression profiles might provide additional insights into the mechanism behind the poor responsiveness of virus-specific CD8\(^+\) T cells to IL-7 and IL-15 during chronic viral infections. These data suggest that CD8\(^+\) T cell exhaustion is accompanied by perturbations in the signaling apparatus available to communicate extracellular information via both the TCR and cytokine receptors to the nucleus.

**Effector Function**

Exhausted CD8\(^+\) T cells have a severe defect in cytokine production including IFN-\(\gamma\) (see Figure 1). It was, therefore somewhat surprising that exhausted CD8\(^+\) T cells continued to express IFN-\(\gamma\) mRNA in amounts that were intermediate between effector and memory CD8\(^+\) T cells (Table 1). Exhausted CD8\(^+\) T cells are also poorly cytotoxic ex vivo (Wherry et al., 2003; Zajac et al., 1998), but granzyme B mRNA remains expressed by exhausted CD8\(^+\) T cells (Table 1). Perforin mRNA is upregulated in effector and memory CD8\(^+\) T cells compared to naive CD8\(^+\) T cells, but not in exhausted CD8\(^+\) T cells (Table 1). This observation suggests that a deficiency in perforin expression could underlie poor ex vivo cytotoxicity by exhausted CD8\(^+\) T cells. In addition, the expression of several genes involved in vesicle transport (down: Snn4, Snx10, and Rabac1) and/or regulation of cytoskeleton (down: Tubb5, Actn1, Macf1, and Actb) was altered in exhausted CD8\(^+\) T cells (Table 1 and Tables S2 and S3). It is possible that changes in vesicle trafficking or cytoskeletal rearrangements impact the generation or release of properly formed lytic granules. In contrast, Fasl mRNA expression was substantially elevated by exhausted CD8\(^+\) T cells compared to naive, effector, and memory CD8\(^+\) T cells, and this pathway might provide an alternate cytolytic mechanism during chronic infection. Together, however, poor IFN-\(\gamma\) production and cytotoxicity by exhausted CD8\(^+\) T cells cannot be solely attributed to lack of IFN-\(\gamma\) or granzyme B gene expression.

**Chemokines and Migration**

Exhausted CD8\(^+\) T cells displayed upregulation of several chemokine genes including Ccl3 (Mip1\(\alpha\)), Ccl4 (Mip1\(\beta\)), and Cxcl10 (IP-10) compared to effector and memory T cells. Ccl5 (Rantes) expression was similar in exhausted and memory CD8\(^+\) T cells, both of which expressed slightly lower amounts of this mRNA compared to effector cells. Thus, even in the absence of robust effector function, exhausted CD8\(^+\) T cells might retain the ability to “sound the alarm” in an attempt to recruit more effective antiviral T cells to the sites of active viral replication. Future studies will be necessary for determining the importance of these elevated chemokine mRNA levels in the in vivo response of exhausted CD8\(^+\) T cells.

Exhausted CD8\(^+\) T cells also have altered expression of molecules involved in cell adhesion (up: Itgax [CD11c], neurophilin, Itga4 [CD49d], Itgb1 [CD29], CD166, Itgb2 [CD18], and Itgav [CD51]; down: Lcam2, Itgase, Sema4d, and Itgb7) and migration and chemotaxis (up: Ccr5, Cxcr3, Cxcr2; down: Edg6, Ccr7, CD62L), suggesting possible changes in migratory properties in vivo. Indeed, several studies have documented altered tissue distribution of virus-specific CD8\(^+\) T cells during chronic infections (He et al., 1999; Wherry et al., 2003).
Transcription Factors in CD8⁺ T Cell Exhaustion

Transcription factors and genes involved in transcriptional regulation was another group of mRNAs substantially altered by exhausted CD8⁺ T cells. This set of genes included at least 15 mRNAs that were uniquely or selectively upregulated and 11 that were downregulated in exhausted CD8⁺ T cells. These observations are consistent with the notion that exhausted CD8⁺ T cells arise from an altered pattern of differentiation compared to functional effector and memory T cells.

PBX3 was the most differentially expressed transcription factor in exhausted CD8⁺ T cells (Table 1 and Tables S2 and S3). PBX3 is a TALE class homeodomain transcription factor implicated in developmental regulation (Sagerstrom, 2004), but the role of PBX3 in CD8⁺ T cell differentiation has not been explored.

A second transcription factor that was of considerable interest and that was revealed by the gene-expression profiles is Blimp-1 (Prdm1). The increased expression of Blimp-1 in virus-specific effector and exhausted CD8⁺ T cells is intriguing, given the role of this transcription factor in regulating terminal differentiation in germinal center B cells (Calame, 2006). Recent studies have revealed an important role for Blimp-1 in regulating T cell responses (Kallies et al., 2006; Martins et al., 2006), but precisely how this transcriptional repressor controls T cell differentiation remains unclear. It will be of considerable interest to investigate how Blimp-1 regulates virus-specific CD8⁺ T cell differentiation during chronic viral infection.

Several additional transcription factors were also revealed by the gene-expression profiles of exhausted CD8⁺ T cells including several upregulated (e.g., Eomes, NFATc1, Jak3, nurr1, and Maf) and downregulated (e.g., Fos, Fosb, junb, myb, and Myc) molecules of potential immunological interest. Eomes is of note given the influence of this molecule on the transcriptional regulation of effector genes (e.g., granzyme B and IFN-γ) and cd122 (Intlekofer et al., 2005; Pearce et al., 2003). Several Klf family members are also downregulated in exhausted CD8⁺ T cells including Klf2, Klf3, Klf4, and Klf13. In particular, changes in Klf2 expression might be important in the exhausted CD8⁺ T cells because Klf2 can regulate T cell quiescence and migration (Carlson et al., 2006; Kuo et al., 1997). Together, these findings show that the expression of genes involved in regulating transcription in exhausted CD8⁺ T cells highlights the altered pattern of differentiation in these T cells.

Metabolic Defects in Exhausted CD8⁺ T Cells

Metabolic and bioenergetic deficiencies in exhausted CD8⁺ T cells were among the most striking patterns that emerged from the gene-expression profiles. A large number of ribosomal subunits were transcriptionally downregulated in exhausted CD8⁺ T cells compared to naive, effector, and memory T cells (Table 1 and Tables S2, S3, and S5). Up to 17 ribosomal subunits, three elongation factors, and four initiation factors were downregulated in exhausted CD8⁺ T cells.

In addition to translational defects, the gene-expression profiles also suggest other fundamental metabolic and bioenergetic changes during CD8⁺ T cell exhaustion. For example, a number of genes involved in energy metabolism and the citric-acid cycle were transcriptionally downregulated in exhausted CD8⁺ T cells but not effector or memory CD8⁺ T cells including Acas21, Sdha, Adcy7, Pdha1, and Acadm. Other metabolic pathways transcriptionally altered in exhausted CD8⁺ T cells included solute and ion channels and aquaporins (Table 1 and Tables S2, S3, and S5). These observations suggest that bioenergetic deficiencies in exhausted CD8⁺ T cells might impact functional responsiveness and perhaps the long-term fitness and survival of this CD8⁺ T cell population.

Other Genes and Pathways of Interest

Several other major classes of mRNAs were altered in exhausted CD8⁺ T cells. These include changes in expression of genes involved in glycosylation, increases in IFN-responsive genes, and several protease mRNAs including upregulation of serpins, some of which can protect from T cell-mediated cytotoxicity (Liu et al., 2004). Several caspases (1, 3, and 4) were upregulated, and Bcl-2 family members were upregulated and downregulated. The precise balance of these proapoptotic and antiapoptotic molecules is difficult to infer simply from the mRNA-expression profiles. However, the mRNA profiles suggest that the regulation of apoptosis is likely to be quite different in the exhausted CD8⁺ T cells compared to naive, effector, and memory CD8⁺ T cells. Many cell-cycle genes were changed in exhausted CD8⁺ T cells, and these changes are predominantly consistent with increased expression of checkpoint proteins and reduced expression of proapoptotic signals.

Several specific genes are also worth noting simply on the basis of their expression profile including plekstrin (up ~50- and ~60-fold in exhausted and effector CD8⁺ T cells), Folate receptor 4 (up in exhausted), and Tmem2 (up in exhausted) (Table 1 and Table S5). Finally, KLRG1 was the most effector-biased gene when exhausted and effector CD8⁺ T cells were directly compared to each other (Table S2).

Progression of CD8⁺ T Cell Dysfunction during Exhaustion

It was of interest to understand how CD8⁺ T cell exhaustion and the expression of markers of T cell differentiation change during the course of chronic infection and development of exhaustion. To address this issue, we performed a kinetic analysis of both functional exhaustion and phenotypic differentiation of virus-specific CD8⁺ T cells after infection with either Arm or clone 13. At day 6 p.i. GP33-specific CD8⁺ T cells in both Arm- and clone 13-infected mice were functional, and a similar proportion of tetramer⁺ CD8⁺ T cells was capable of producing IFN-γ during both infections (Figures 3A and 3B). Although there were slightly fewer IFN-γ and TNF-α coproducers during clone 13 infection at day 6, the amount of IFN-γ, TNF-α, and IL-2 produced (MFI) by the virus-specific CD8⁺ T cells...
after either infection was very similar (Figure 3B). Over the ensuing weeks after clearance of infection, virus-specific CD8+ T cells generated during acute infection all remained functional (all tetramer+ cells produced IFN-γ), and in fact, the percentage cells coproducing multiple cytokines (IFN-γ + TNF-α and IFN-γ + IL-2 coproducers) and the amount of cytokine produced per cell (MFI) increased. In contrast, during chronic LCMV infection, virus-specific CD8+ T cells became progressively less functional over time. By 2–3 weeks p.i., 50% or more of the tetramer+ cells could not
produce IFN-γ, and the frequency of cytokine co-producers and amount (MFI) of individual cytokines produced per cell declined dramatically (Figure 3B). Thus, although virus-specific CD8+ T cells were initially functional after both Arm and clone 13 infections, during chronic LCMV infection, virus-specific CD8+ T cells become progressively less functional (i.e., exhausted) after the first week of infection.

Altered T Cell Differentiation during Exhaustion

We next profiled the phenotypic changes in virus-specific CD8+ T cells during the progression to exhaustion versus development of memory by using a set of genes identified by microarray analysis. At day 6 p.i., the inhibitory receptors PD-1, LAG-3, and 2B4 were upregulated on DbGP33-specific CD8+ T cells during both Arm and clone 13 infections (PD-1 was perhaps slightly higher during clone 13 infection at this time point.). However, by day 8 p.i., the expression of these inhibitory receptors was low during Arm infection and remained low thereafter, consistent with the clearance of the virus. In contrast, during chronic infection, PD-1, LAG-3, and 2B4 remained highly expressed, and especially for PD-1 and 2B4, the expression increased on virus-specific CD8+ T cells after day 8 (Figures 4A and 4B). Thus, expression of inhibitory receptors is not unique to chronic viral infection. Rather, during initial T cell activation during both acute and chronic infection, these pathways are upregulated; yet, at these early time points, T cells are functional. It is possible that early during infection signals from positive costimulatory pathways outweigh those from negative pathways. Later, during chronic infection, the persistent (and increased) expression of inhibitory receptors might shift the balance toward inhibition of T cell function. Future studies will be necessary for determining precisely when and how these inhibitory pathways control antiviral T cell function in vivo.

At early times p.i. (day 6), virus-specific CD8+ T cells primed during acute versus chronic infection expressed a similar pattern of activation and differentiation markers including CD44, CD25, granzyme B, 1B11, CD62L, CD27,
CXCR3, and Ly6C. In contrast to acute infection, however, virus-specific CD8+ T cell expression of granzyme B and 1B11 remained elevated during chronic infection, whereas CD62L remained low. The activation marker CD69 was low at day 6 (Figure 4C) after both Arm and clone 13 infection despite the presence of antigen. To examine whether CD69 was expressed earlier during infection, we used a P14 adoptive transfer approach to visualize virus-specific CD8+ T cells at day 3 p.i. At this time point, CD69 expression was elevated after both Arm and clone 13 infections, and a similar profile of CD69 expression versus cellular division was observed (Figure 4C). During clone 13 infection, CD69 again began to increase after day 12 and was uniformly high on virus-specific CD8+ T cells from chronically infected mice after day 21. The reasons for this dynamic regulation of CD69 are not clear but suggest that there might be a period of time early during infection when T cells are refractory to further upregulation of CD69.

Although CD44 expression was elevated compared to naive CD8+ T cells after both infections, beginning ~3 weeks p.i., CD44 expression was lower on exhausted CD8+ T cells compared to memory CD8+ T cells consistent with the microarray data (Table S3). CD27 is of interest because this molecule has been used extensively to characterize human antiviral CD8+ T cells responding to different persisting infections (van Lier et al., 2003). Although two subsets of antiviral CD8+ T cells were clearly present on the basis of CD27 expression during the first ~3-4 weeks after acute infection, only CD27hi cells were found during chronic infection. Memory CD8+ T cells also eventually all became CD27hi. Other molecules including CXCR3 and Ly6C also displayed dynamic expression patterns that differed between the development of memory (CXCR3hiLy6Chi) and exhausted (CXCR3intLy6Clo) CD8+ T cells.

The expression of the cytokine receptors CD122, CD124, and CD127 was low on exhausted CD8+ T cells at late time points during chronic infection compared to memory CD8+ T cells. CD124 and CD127 were permanently downregulated early after infection in contrast to the re-expression of these cytokine receptors after acute infection (Figure 4C and Figure S2). CD122, on the other hand, was similar after acute or chronic infection at day 6, was higher on exhausted CD8+ T cells from day 8 to day 21, but eventually became lower in exhausted compared to memory CD8+ T cells at later times p.i. The expression pattern of these receptors is consistent with the gene-expression data (Table 1 and Tables S2, S3, and S5) and suggests that exhausted CD8+ T cells will not efficiently respond to IL-2, IL-4, IL-7, and IL-15 at later times p.i.

KLRG1 was one of the few molecules examined that differed in protein expression at early time points after infection. During clone 13 infection, virus-specific CD8+ T cells expressed lower amounts of KLRG1 at day 6 (and at all subsequent time points) compared to virus-specific CD8+ T cells generated after Arm infection. Expression of KLRG1 by CD8+ T cells has been associated with repetitive antigen stimulation, cellular senescence, and terminal differentiation (Joshi et al., 2007; Masopust et al., 2006; Voehringer et al., 2001). The KLRG1lo phenotype of exhausted CD8+ T cells was, therefore, a bit surprising because these cells have a poor proliferative capacity upon antigen stimulation (Wherry et al., 2004; Wherry et al., 2005). It will be interesting to determine how the relationship between KLRG1 expression and proliferative potential applies to chronic infection during which the T cells experience continuous rather than intermittent antigen stimulation.

Finally, virus-specific CD8+ T cells were larger in size during chronic infection compared to acute infection between days 8-15. This difference likely reflects persisting TCR stimulation during clone 13 infection, but not following Arm infection at these time points. However, exhausted CD8+ T cells were significantly smaller than memory CD8+ T cells at later time points (Figures 4A, 4B, and 4D). This reduced cell size for exhausted CD8+ T cells is one of the last differences to emerge. Cell size is an excellent indicator of metabolic fitness, and when metabolism is compromised, lymphocytes become considerably smaller (Rathmell et al., 2000). The observations on the size of exhausted compared to memory CD8+ T cells are in good agreement with the gene-expression profile of metabolic fitness described above.

Gene-Expression Profile of Exhaustion Is Distinct from Anergy

Exhaustion of antigen-specific CD8+ T cells has been described during a number of both experimental and human infections. Anergy also results in T cell dysfunction. Anergy occurs when initial TCR signals are received in the absence of optimal costimulation leading to a state of hyporesponsiveness (Schwartz, 2003). A transcriptional signature of anergy has been defined, resulting in the identification of 14 anergy-associated genes (Macian et al., 2002). In this study, anergized T cells were refractory to subsequent anti-CD3 stimulation and had decreased transcription of several cytokine genes including Il2, Il10, Tnf, and Ccl3. The expression profile of anergy-associated genes was also examined with an in vivo tolerance model in which many of the anergy-associated genes identified in vitro were confirmed, thus defining a molecular signature of anergy both in vitro and in vivo (Macian et al., 2002).

To examine whether exhaustion and anergy shared similar transcriptional signatures, we examined the expression pattern of the 14 anergy associated genes identified by Macian et al. (2002) in the exhausted CD8+ T cells. Few of the anergy-associated genes were upregulated in exhausted CD8+ T cells (Figure 5A). Also, this panel of 14 genes did not clearly distinguish exhausted CD8+ T cells from effector or memory CD8+ T cells. In this type of analysis, the expression of individual anergy-associated genes was assessed relative to a threshold value (i.e., ±2-fold). It remained possible that, although the anergy-associated genes were not individually overexpressed >2-fold in exhausted CD8+ T cells, these genes as a set might be specifically enriched during exhaustion.

To test this possibility, we next used gene-set-enrichment
analysis (GSEA; [Subramanian et al., 2005]) (Figure 5B). GSEA is performed by assigning a rank order to the expression of the ∼9000 genes from the U74A arrays (ESTs are excluded from this analysis.). The location of each gene of interest is then determined in the rank-ordered data set, and the specific enrichment of the set of genes at the higher (or lower) ends of the rank order is determined. As shown in Figure 5B, the anergy-associated gene set did not show specific enrichment in the exhausted CD8+ T cells, and the “hits” for the individual genes were distributed essentially throughout the rank order and not accumulated on the left side as is expected for enriched genes (Subramanian et al., 2005). Taken together, the data in Figure 5 suggest that CD8+ T cell exhaustion examined in the current study is distinct from the process of anergy profiled in previous studies (Macian et al., 2002). Thus, T cell exhaustion and anergy, at least for these two models, do not appear to share the same transcriptional signature.

DISCUSSION

A number of pathways, identified in the present study, are associated with CD8+ T cell exhaustion during chronic viral infection. One of the most obvious patterns that emerged was the overexpression of cell-surface inhibitory molecules by virus-specific CD8+ T cells during chronic infection. Our previous observations on the role of PD-1
in regulating exhaustion (Barber et al., 2006) suggest that the group of inhibitory pathways identified in the current study might provide additional targets for investigation. It will be important to determine whether inhibitory pathways such as LAG-3, 2B4, and GP49B have distinct or overlapping roles in regulating T cell responses during chronic infection. A puzzling issue is why exhausted CD8+ T cells would express multiple receptors with inhibitory function. One possibility is that these are redundant pathways for ensuring adequate control of immunopathology. A second possibility is that these different receptors have nonoverlapping intracellular signaling targets. A third possibility is that the expression of the ligands for these receptors is a key to their effective inhibition of T cell responses. The ligands for the inhibitory receptors overexpressed by exhausted CD8+ T cells include a diverse array of both cell-surface and soluble molecules expressed in a restricted (e.g., professional APC only) or broadly distributed manner. A combination of these mechanisms might explain the apparent redundancy of inhibitory receptors. Future studies are aimed at investigating the roles of these different pathways in regulating CD8+ T cells during chronic viral infections.

A second novel observation revealed by the transcriptional profiles of exhausted CD8+ T cells was the dramatic alteration in metabolic and bioenergetic pathways compared to the pathways of naive, effector, and memory T cells. Exhausted CD8+ T cells downregulated components of the translational machinery, molecules involved in the citric-acid cycle, and energy metabolism, and these cells also became considerably smaller in size. One implication of downregulated ribosomal subunits is that these cells might have difficulty producing the quantity of cytokines or other effector proteins normally produced by functional effector and memory CD8+ T cells. This interpretation would also fit with the predominantly intact expression of the mRNA for such as IFN-γ and granzymes B, A, and K.

Cell size is a useful indicator of metabolic fitness (Rathmell et al., 2000), suggesting that the reduction in cell size for exhausted CD8+ T cells reflects some of the metabolic deficiencies observed in the gene-expression profiles. It is interesting to note that the inhibitory receptors PD-1 and CTLA-4 can impair optimal AKT activation, a key signaling pathway for glucose utilization and cellular metabolism (Parry et al., 2005). Impaired translational machinery and metabolic fitness is also likely to impede robust proliferation. Production of a new complement of cellular proteins and the increase in cell size necessary to give rise to two daughter cells during mitosis might be substantially compromised by the bioenergetic and ribosomal deficiencies present in exhausted CD8+ T cells. It should be noted, however, that these metabolic changes do not universally prevent protein translation because a number of molecules, such as the inhibitory receptors, are upregulated in exhausted CD8+ T cells at the protein level. It will probably be important to address these bioenergetic and translation deficiencies in therapeutic strategies aimed at restoring optimal function from exhausted T cells.

Kinetic profiling of T cell dysfunction and differentiation during chronic infection confirmed the expression pattern of many proteins predicted by the gene-expression profiles. More importantly, this kinetic analysis demonstrated the progressive nature of CD8+ T cell exhaustion. Of the 22 properties examined by flow cytometry (including cytokine coproduction), only three differed between virus-specific CD8+ T cells from Arm and clone 13 infection at day 6 p.i. (TNF-α + IFN-γ coproduction, PD-1, and KLRG1). By day 8 p.i., nine differences were apparent. After day 8 of acute infection, virus-specific CD8+ T cells continued to progressively differentiate into memory T cells and acquired new phenotypic and functional characteristics such as IL-2 production. T cell exhaustion, in contrast, followed an altered pattern of differentiation, and after day 8 of infection, the differences between functional memory T cell differentiation and exhaustion increased. Between 1–2 weeks p.i., 18 of 22 properties profiled differed, and after ~1 month, this had increased to 20 or 21 differences out of 22 properties examined. Thus, our results indicate that CD8+ T cell exhaustion is a graded process during chronic viral infection and that there are stages in the differentiation and development of exhausted CD8+ T cells. Such a graded or progressive development of CD8+ T cell exhaustion might have implications for therapeutic strategies that could be tailored to overcome specific defects of a particular stage of exhaustion.

Our data also suggest that T cell anergy and exhaustion occur by distinct molecular mechanisms. In addition to the anergy-associated gene set identified by Macian et al. (2002), several other genes have been reported to be associated with T cell anergy including Egr-2, Egr-3 (Harris et al., 2004; Safford et al., 2005), and grail (Mueller, 2004). These genes were not selectively expressed by exhausted CD8+ T cells compared to functional effector and memory CD8+ T cells. Recent evidence suggests that the proper NFAT:AP-1 ratio promotes T cell activation, whereas NFAT activity in the absence of AP-1 favors anergy (Macian et al., 2002). Indeed, NFATc1 is transcriptionally elevated in exhausted CD8+ T cells, and Fos (part of the AP-1 dimer), Fosb, and JunB are downregulated in exhausted CD8+ T cells compared to memory CD8+ T cells. Thus, some alterations in the NFAT:AP-1 balance might exist in exhausted CD8+ T cells compared to memory CD8+ T cells, but the lack of expression of target genes of AP-1-independent NFAT transcription such as E3 ubiquitin ligases (Heissmeyer et al., 2004) suggest that the major transcriptional mechanisms of anergy and exhaustion are predominantly distinct.

Mouse models including LCMV have been invaluable in dissecting T cell responses to chronic viral infections. For example, recent studies on the role of the PD-1 pathway in regulating CD8+ T cell exhaustion first during LCMV, and soon thereafter for HIV, HBV, and HCV infection highlight the translation of observations from mouse models to human viral infection (Barber et al., 2006; Boni et al., 2007; Day et al., 2006; Petrovas et al., 2006; Radziewicz et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). It will be important in the future to determine whether additional
features of the molecular signature of CD8+ T cell exhaustion in the LCMV model can also be extended to humans. Recent studies suggest that the transcriptional profile of HIV-specific CD8+ T cells from humans might indeed share similarities to exhausted LCMV-specific CD8+ T cells (W.N.H. and T.R. Golub, unpublished data). Future studies will be necessary to determine the therapeutic implications of pathways revealed by transcriptional profiling of mouse and human CD8+ T cells responding to chronic viral infections.

In summary, these studies provide a framework with which to begin dissection of the factors that influence virus-specific CD8+ T cell differentiation during chronic infections. In addition, these studies identify a number of inhibitory, signaling, and metabolic pathways that might underlie the functional defects in these CD8+ T cells and might also represent novel targets for immunotherapy.

EXPERIMENTAL PROCEDURES

Mice and Infections
Four- to six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory. We used the LCMV-specific P14 TCR transgenic system to generate effector and memory CD8+ T cells as described (Kaech et al., 2002). In brief, P14 chimeras were made by adoptive transfer of a small number of naive P14 CD8+ T cells (~6 x 10^5) to naive congenic recipient mice followed by LCMV infection. For acute infections, mice were infected with 2 x 10^6 pfu of LCMV Arm i.p. For chronic infections, mice were given 2 x 10^6 pfu of LCMV clone 13 i.v. and depleted of CD4+ T cells with 200 x 10^4 GK1.5 on days -1 and +1 p.i. Viral titers in chronically infected mice were verified by plaque assay in the serum prior to use. Plaque assays were performed as previously described (Wherry et al., 2003).

Lymphocyte Isolation, Flow Cytometry, and Functional Analysis
Lymphocytes were isolated and MHC class I peptide tetramers were generated and used as previously described (Wherry et al., 2003). All antibodies were obtained from BD Bioscience (San Diego, CA) except for granzyme B and KLRG1 (Caltag). Intracellular cytokine staining was performed as previously described (Wherry et al., 2003). Elisas were performed to verify technical replicates and their quality. Data analysis and clustering was done in gene-chip-analysis software GeneSpring GX 7.3 (Agilent). We performed quality-control checks with R-pack (BioConductor) to verify technical replicates and their quality. Data analysis and clustering was done in gene-chip-analysis software GeneSpring GX 7.3 (Agilent). The data were preprocessed with RMA (Robust Multichip Average) normalization. There were four technical replicates for each cell type, naive, memory, and exhausted, and three for effectors. Technical replicates were averaged for fold-change study. RMA expression values for genes upregulated and downregulated by 2.0-fold change between each cell types were exported from Genespring to Microsoft Excel and analyzed further. We used K means clustering with Pearson correlation to understand how similar or distant each of the cell types were from one another with respect to naive. RMA expression values with 1.4-fold cutoff between all four cell types were used as input for pathway analysis in Ingenuity pathway analysis software (Ingenuity Systems). GSEA was performed as described (Subramanian et al., 2005).

Supplemental Data
One figure and five tables are available at http://www.immunity.com/cgi/content/full/27/4/670/DC1/

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**Accession Numbers**

The gene-expression data described in this paper have been deposited in the NCBI’s Gene Expression Omnibus and are accessible through GEO accession numbers GSM235537-GSM235551.